

PIAS3 (protein inhibitor of activated STAT-3) modulates the transcriptional activation mediated by the nuclear receptor coactivator TIF2

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Abstract PIAS3, a member of the protein inhibitor of activated STAT family, was found to interact *in vivo* and *in vitro* with TIF2, a previously described coactivator for nuclear receptors. The interaction is mediated by two distinct non-contiguous regions of TIF2. We found that TIF2–PIAS3 interaction occurs through a unique domain of PIAS3, very rich in acidic residues and conserved throughout the PIAS family. PIAS3 modulates the ability of TIF2 to mediate ligand-enhanced transcription activation positively or negatively, for different steroid receptors. Taken together, our results indicate a potential role of PIAS3 as transcriptional modulator of TIF2-mediated signalling. © 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: TIF2; Nuclear receptor; Protein inhibitor of activated STAT-3; Transcriptional modulator

1. Introduction

Nuclear receptors (NRs) comprise a family of transcription factors that regulate gene expression in a ligand-dependent manner. The NR superfamily includes receptors for steroid and thyroid hormones, retinoids, vitamin D, as well as receptors for fatty acids, prostaglandins and intracellular metabolites [1,2]. NRs can activate or repress target genes by binding to cognate DNA response elements as homo- or heterodimers. Ligand binding induces a transconformation of helix H12, a short amphipathic helix in the carboxy-terminus of the NR ligand binding domain, which in the agonist conformation contributes to the surface required for the interaction with transcriptional coactivators. Helix H12 is thus an integral part of the transcription activation function 2 (AF2) that is induced by agonists [3]. Concomitantly with agonist binding the interface established between co-repressors and some hormone-free NRs is destabilized. Binding of coactivators and co-repressors to the NRs results in the assembly of distinct multiprotein complexes which exert, respectively, histone acetyltransferase (HAT) and histone deacetylase (HDAC) activity. It is believed that acetylation of histone tails results in decondensation of chromatin which may be a prerequisite, or is sufficient, for transcriptional activation by agonist-bound NRs. Acetylation and deacetylation of non-histone proteins

and other transcription factors have been reported to be mediated by the HAT and HDAC complexes, but the significance of these events in the context of NR signalling has remained elusive. In addition to HAT and HDAC complexes, other multiprotein complexes such as SWI/SNF and TRAP/SMCC/DRIP/ARC complexes have been shown to be involved in NR signalling. The precise temporal order of action and detailed implication of these various complexes are still not well understood (see for review [4–8]).

Members of the p160 coactivators, such as human SRC-1, TIF2 and AIB1, contain two different transcription activation domains (AD1 and AD2) in the carboxy-terminus. AD1 acts through recruitment of the CREB binding protein (CBP) or the CBP-related protein p300 [9]. Additional factors, some of which exhibit methyltransferase enzymatic activity, can be recruited to the AD2 of p160 coactivators [10–12]. The p160 family of nuclear receptor coactivators contains a highly conserved amino-terminal basic helix-loop-helix (bHLH)/PAS domain that is also present in members of the Per/Arnt/Sim (PAS) family of transcription factors and mediates protein–protein interactions. The role of this putative protein dimerization motif in the context of the p160 family factor remains to be established.

In mammals, PIAS proteins were first discovered as transcriptional co-regulators of the JAK-STAT pathway [13]. The binding of cytokines to cell surface receptors activates the Janus family of tyrosines kinases (JAK), which phosphorylate a family of at least seven cytoplasmic transcription factors termed STAT (signal transducer and activator of transcription). PIAS1 (protein inhibitor of activated STAT-1) was identified as a specific inhibitor of STAT-1 signalling, but conversely can enhance the transcriptional activity of steroid hormone receptors [13–15]. In vertebrates, five PIAS proteins (PIAS1/GPB, PIAS3, ARIP3/PIASx α , Miz/PIASx β and PIASy) are known [13,14,16]. All PIAS proteins have been reported to coactivate certain steroid receptor-dependent transcriptions. This effect varied depending on the steroid receptor, the promoter, and the cell type studied [13,14,16,17]. PIAS1 and PIAS3 inhibit DNA binding of the STAT-1 and STAT-3 factors [13,18]. In contrast, the PIASy protein represses the transcriptional activity of STAT-1 and the androgen receptor without affecting their DNA binding ability [15]. In *Drosophila*, the PIAS ortholog dPIAS cooperates with stat92E to regulate eye and blood cell development, presumably by transcriptional regulation [19]. However, an even more general role for PIAS family protein was suggested by the characterization of the suppressor of position effect vari-

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egation, Su(var)2–10 (or ZIMP), as a *Drosophila* PIAS [20]. Deficiencies in Su(var)2–10 caused strong effects on both mitotic chromosome condensation and the organization of interphase chromatin. In this regard, it has recently been reported that PIASy regulates the activity and subnuclear localization of the Wnt-responsive transcription factor LEF1 by directing SUMO (small ubiquitin-related modifier) addition to LEF1 [21]. Notably, PIASy binds to nuclear matrix-associated DNA sequences and targets LEF-1 to nuclear bodies, suggesting that PIASy-mediated subnuclear sequestration accounts for the repression of LEF1 activity.

To understand the molecular mechanism of TIF2 functions and identify potential associated proteins, we performed a yeast two-hybrid screen using the amino-terminal region of TIF2, containing the bHLH/PAS domain as bait. We identified PIAS3 as a protein that interacts specifically with TIF2 in vivo and in vitro.

2. Materials and methods

2.1. Two-hybrid screening

The TIF2 deletion mutant containing amino acids 1–624 was cloned in phase 3' of the DNA binding domain of LexA in pBTM116 to produce a bait fusion protein. A mouse embryo (9.5–12.5 dpc) cDNA library in the λ pASV3 phage yeast vector was used for screening [22]; amplification and plasmid excision (*cre-lox* system) was done in *Escherichia coli* BNN132. After plasmid excision cDNAs are expressed as VP16 acidic activation domain fusions. Two-hybrid screening [23] was done by sequential transformation of bait and library vectors in *Saccharomyces cerevisiae* L40 α carrying two genomically integrated reporters, the *lexA-HIS3* and *lexA-LacZ*. After transformation, yeast were plated on histidine-lacking selective media containing 30 mM 3-amino-1,2,4-triazole and incubated at 30°C until transformants appeared. Transformants that grew more rapidly were restreaked in duplicate in selective medium and tested for β -galactosidase expression. One clone (N256) was selected for further analysis. Database searches using the N256 sequence showed that N256 is identical to the mouse PIAS3.

2.2. Plasmids

All recombinant DNA work was performed according to standard procedures. VP16-PIAS3 and VP16-N256 were constructed by cloning a *Bam*HI fragment containing full-length PIAS3 cDNA or the clone isolated in the two-hybrid screening into the *Bam*HI site of vector pSG5-NVP16-LB3. pSG5-NVP16-LB3 contains a cassette expressing a nuclear localized VP16 AAD in the context of the pSG5 vector [24]. VP16-PIAS3 Δ (340–482) was constructed by homologous recombination. Briefly, the N-terminal part of PIAS3(1–340) was amplified by PCR using the oligos 5'-GTACGGTGGGGGGCTACGGAGATCTGCCACCATGGTGATGAGTTTCCGAGTGTCTG-3' (sense) and AGAAAATAAATCTAAACCTTGTAACCATCAATAATCAGCGATTTCATAG-3' (antisense). The oligos 5'-CGCTGATTATTGATGTTCACAGGTTTAGATTATTTCTTTCTTCCTTCAG-3' (sense) and 5'-AACAAGTTCTGCTTTAATAAGATCTTCAGTCCAGG-GAAATGATGTCTGAC-3' (antisense) were used to amplify the C-terminal fragment of PIAS3 (amino acids 482–585). N- and C-terminal fragments of PIAS3 containing homologous sequences for recombination in 3' and 5' respectively were used to transform competent bacteria together with the vector pSG5-NVP16-LB3 linearized at the *Bgl*II site.

The TIF2 cDNA expression vector was previously described [25]. The GAL4-TIF2 chimera was constructed by PCR amplification of the full-length cDNA of TIF2 followed by subcloning in PG4MpolyII [26]. For in vitro binding assays, the indicated regions of TIF2 were fused to GST in the pGEX-2TK plasmid (Pharmacia) or to a tag of six histidines in the pCalKc plasmid (Stratagene). The reporter plasmid (17m)₅-TATA-CAT [27] contains five copies of the GAL4 response element in front of a simple TATA motif upstream of the CAT reporter gene. Mouse mammary tumor promoter CAT construct was described previously [28]. Receptor cDNAs used in this study encode the human glucocorticoid (HG1), androgen (hAR) and progesterone (hPR1) receptors. Details concerning the plasmid construc-

tions, all of which were verified by sequencing, are available on request.

2.3. Pull-down assays with [³⁵S]methionine-labelled proteins

DNA was transcribed and translated in vitro using the TNT T7 coupled reticulocyte lysate system (Promega) following the instructions of the manufacturer. The reticulocyte lysate containing ³⁵S-labelled protein was then incubated as described [29] with purified 6 \times His-tagged or GST-linked TIF2 peptides [30]. Bound proteins were recovered in SDS sample buffer and revealed by fluorography of SDS-polyacrylamide gels.

2.4. Cell culture and transient transfection

COS-1 cells were maintained in Dulbecco's minimal essential medium containing penicillin (25 U/ml), streptomycin (25 U/ml), and 10% fetal bovine serum. Transient transfections were performed using the calcium phosphate co-precipitation protocol in the presence of phenol red-free medium containing charcoal-treated serum. At 14–16 h after transfection, COS-1 cells were exposed to dihydrotestosterone, dexamethasone, or progesterone for 24 h. Transfected cells were lysed and assayed for reporter gene activity using the CAT immunodetection system (Boehringer Mannheim) according to the manufacturer's instruction. β -Galactosidase, measured as previously described [9], was used to normalize variations in the transfection efficiencies.

2.5. Immunoblotting

Whole cell extracts of COS-1 cells were prepared in RIPA buffer and resolved by electrophoresis on 12% polyacrylamide gels under denaturing conditions (SDS-PAGE). Proteins were electroblotted onto nitrocellulose transfer membrane (Schleicher and Schuell). Membranes were incubated with antibodies against VP16 (IGBMC monoclonal antibody service). Horseradish peroxidase-conjugated anti-mouse immunoglobulin G antibody (Ozyme) was used as the secondary antibody. Immunocomplexes were visualized with Western blotting detection reagents according to the manufacturer's instructions (Pierce).

3. Results

3.1. Identification of PIAS3 as an interacting partner for the nuclear receptor coactivator TIF2 in a yeast two-hybrid screening

TIF2, a member of the p160 coactivator family, mediates the transcriptional signalling of nuclear receptors. To gain further insight into the regulatory mechanisms of TIF2-dependent signalling cascade(s), we searched for additional interaction partners of TIF2. Toward this end, we performed a yeast two-hybrid screen with a TIF2-LexA fusion protein. The N-terminal region of hTIF2, TIF2.0, encompassing the first 640 amino acids of the nuclear receptor coactivator was used as a bait in a yeast two-hybrid screening of a mouse embryo cDNA library. Yeast transformants containing the LexA-DBD fused to the first 640 amino acids of TIF2 and mouse proteins fused to the activation domain of VP16 were selected according to their ability to grow in histidine-lacking media. The positive transformants were reselected in a second round for their ability to induce the second reporter gene, *lacZ*, present in the yeast strain L40 α . Sequence analysis revealed that one of the positive clones, called N256, encoded a truncated protein of identical sequence to residues 353–546 of mouse PIAS3.

3.2. TIF2 and PIAS3 interact in mammalian cells

To confirm that the TIF2–PIAS3 interaction observed in yeast cells is relevant in animal cells, we used a mammalian two-hybrid assay. Indeed, the transcriptional activity of GAL4 DNA response element-targeted TIF2 (GAL4-TIF2, comprising the DNA binding domain of GAL and the full-

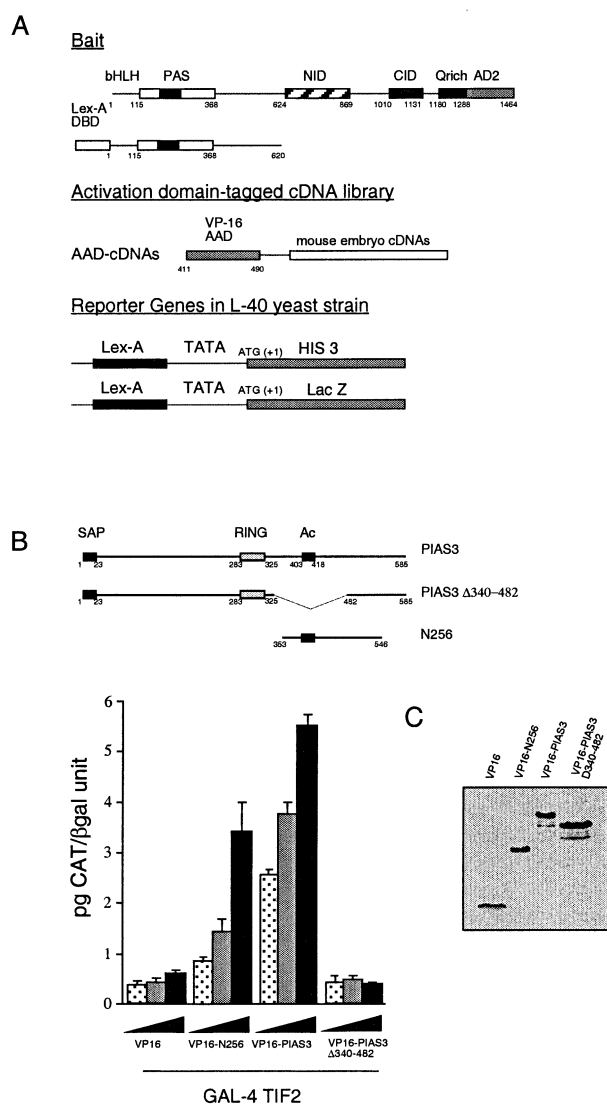


Fig. 1. Isolation of PIAS3 by two-hybrid screening in yeast. **A:** A schematic representation of functional domains of TIF2 as well as the LexA chimera used as a bait. NID, nuclear receptor interacting domain; CID, CBP interacting domain; Q rich, glutamic acid-rich domain. Numbers below the line diagrams indicate the amino acid positions of the respective protein domains. The VP16 AAD-tagged mouse embryo cDNA expression library is represented below. Transcription of the integrated HIS3- and lacZ-based reported genes is regulated by a LexA promoter in the yeast reporter strain L40α. **B:** PIAS3 and TIF2 interact in a mammalian two-hybrid system. Deletion of a region of PIAS3 encompassing the acidic region abolishes the interaction between PIAS3 and TIF2. Schematic line diagrams of PIAS3. PIAS3 contains a putative chromatin binding SAP domain, a RING domain, and a C-terminal acidic domain. COS-1 cells were co-transfected with 300 ng per well of the (17m)₅-TATA-CAT reporter gene, 1 μg of GAL-TIF2 chimera and increasing amounts (25, 50, 100 ng) of N256 and (0.25, 0.5, 1 μg) PIAS3 and PIAS3Δ(340–482) VP16 chimeras as indicated. Each bar represents the mean value obtained from at least three different experiments, standard deviations are indicated. In **C** expression levels of VP16 and VP16-PIAS proteins are shown to demonstrate that they are expressed at similar levels in transient transfection experiments. COS-1 cells were transiently transfected with expression vectors for VP16, VP16-N256, VP16-PIAS wild type and VP16-PIAS3Δ(340–482) in the same conditions as in two-hybrid assays previously shown. After 24 h of incubation the cells were harvested, and Western blot analysis was performed with anti-VP16 antibody.

length TIF2) significantly increased with increasing amount of vectors that expressed the VP16-tagged N256 or full-length PIAS3 proteins (Fig. 1B). We thus conclude that PIAS3 is a novel partner of the TIF2 coactivator.

N256 harbors an acidic region as the sole discernible feature. As this acidic region is also known to be involved in the case of PIAS1/STAT-1 interaction, we tested whether it can also mediate TIF2 binding. For this, we generated a mutant lacking the characteristic acidic region, PIAS3Δ340–482, as a VP16 fusion and we tested its ability to interact with TIF2. In a mammalian two-hybrid system, this mutant did not retain the ability of PIAS3 to interact with TIF2 (Fig. 1B). Equal expression of PIAS3 proteins was confirmed by Western blotting (Fig. 1C). These data indicate that a single domain of PIAS3 that encompasses the acidic region is both necessary and sufficient for the interaction with TIF2.

3.3. TIF2 and PIAS3 interact in vitro

To study whether the observed in vivo interaction between TIF2 and PIAS3 in yeast and mammalian systems is direct or indirectly mediated by other factors, we performed pull-down experiments with various immobilized histidine-tagged TIF2 fragments. The originally isolated cDNA clone, N256, encoding the amino acids 353–546 of PIAS3 and the full-length cDNA of PIAS3 were radiolabelled by in vitro translation, and their interaction with immobilized His-tagged TIF2 was determined. Interestingly, fragments of TIF2, TIF2.0 and TIF2.1, encoding the N-terminal and central part of TIF2 respectively, interacted with both the N256 and the full-length

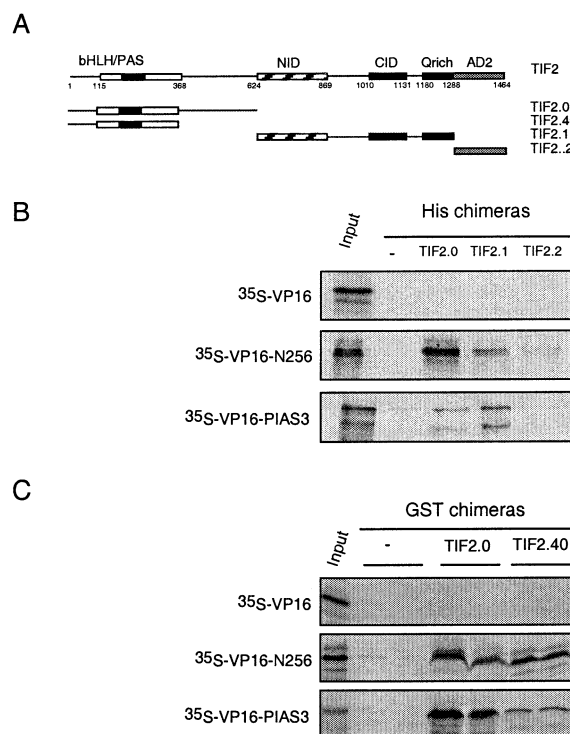


Fig. 2. In vitro mapping of PIAS3 interacting domains within TIF2. **A:** Schematic line diagrams of TIF2 domains and N- and C-terminally truncated TIF2 proteins used in the assay. **B:** N256 and PIAS3 as VP16 chimeras were in vitro translated in the presence of [³⁵S]methionine and incubated with immobilized purified GST- or His-TIF2 fragments in a batch assay. Proteins were resolved by SDS-PAGE, and detected by fluorography. Lane 1 show 1/5 the amount of ³⁵S-labelled protein used in the assay.

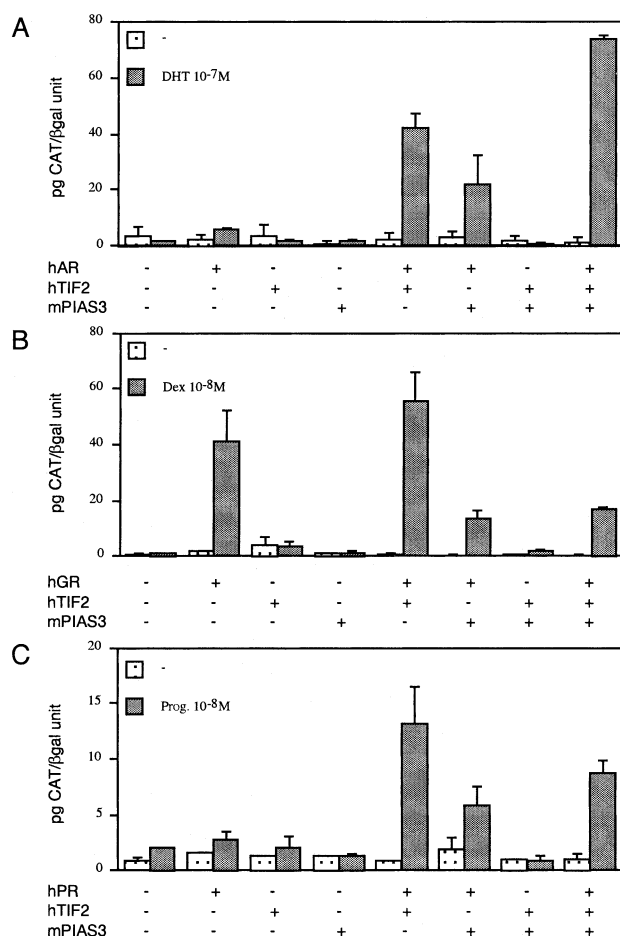


Fig. 3. PIAS3 modulates TIF2 transcriptional activity in a nuclear receptor-dependent manner. COS-1 cells were transfected with 500 ng MMTV-CAT reporter together with 150 ng of androgen (A), glucocorticoids (B) or progesterone (C) receptors with or without 300 ng of TIF2 and PIAS3 in the presence or absence of the indicated ligands. Each bar represents the mean value obtained from at least three different experiments, standard deviations are indicated.

PIAS3 protein. However, the C-terminal part of TIF2 (TIF2.2), which contains the activation domain 2 (AD2) did not interact with these proteins.

To characterize more in detail the observed interaction between the N-terminal part of TIF2 and PIAS3, we studied the *in vitro* interaction between PIAS3 and a fragment encompassing the bHLH/PAS domain of TIF2, TIF2.40, using GST pull-down assays (Fig. 2). The N256 and full-length PIAS3 proteins effectively bound to this N-terminal region of TIF2, which spans residues 1–381. Note that no significant interaction of PIAS3 was observed in these assays with the GST part of the fusion proteins. These results indicate that the bHLH/PAS domain of TIF2 can autonomously interact with PIAS3. Thus, two non-contiguous regions of TIF2, located in the N-terminal (TIF2.0) and in the central (TIF2.1) regions of TIF2 can independently interact with the region that encompasses the acidic domain of PIAS3.

3.4. TIF2 and PIAS3 differentially coactivate androgen, glucocorticoids and progesterone receptors

We have demonstrated previously that TIF2 stimulates the transcriptional activity of several NRs in an agonist- and AF2

integrity-dependent manner. To investigate how PIAS3 could affect TIF2 transcriptional coactivator activity, reporter gene assays were performed using the mouse mammary tumor virus promoter to compare the effect of either PIAS3 or TIF2 alone or both in combination in the transcriptional regulation mediated by AR (Fig. 3). Coexpression of PIAS3 and AR increased the androgen-dependent transcription activity of the MMTV promoter, in a similar manner as cotransfection of TIF2 and AR. Cotransfection of both PIAS3 and TIF2 resulted in an additive enhancement of the androgen response. We also tested the PIAS3 influence on the transcriptional activation by hGR and hPR. To avoid any differences due to promoter context, we used the MMTV-CAT reporter. In contrast to AR-mediated transactivation, coexpression of PIAS3 inhibited glucocorticoid signalling even though TIF2 enhanced glucocorticoid-mediated transcription. PIAS3 enhanced moderately ligand-dependent transactivation by PR, but it negatively modulated PR function when TIF2 was present. Thus, PIAS3 behaved in a receptor-selective fashion, in that its ability to modulate transcription mediated by different steroid receptors varied substantially.

4. Discussion

The N-terminus is the most highly conserved region in the TIF2 protein. Nevertheless, no insight into its function has been obtained so far. The striking homology of the N-terminal region of TIF2 with bHLH/PAS transcription factors suggested to us that the TIF2 N-terminus may establish weak or non-selective DNA contacts and/or protein–protein interaction to stabilize the coactivator complex(es), help to anchor the complex to DNA and/or mediate additional protein–protein interactions required for signalling by the cognate complex. Here, we report the isolation of such an additional interacting partner for TIF2, previously described as mouse PIAS3.

PIAS proteins share several structural elements well conserved along all family members. It has recently been predicted that PIAS proteins harbor a putative DNA binding domain, the SAP (SAF-A, Acinus, PIAS) module [31], at their N-termini (residues 1–23 in PIAS3). The C-terminal region of PIAS3 contains an acidic region (residues 403–418). Deletion of this region abolished the ability of PIAS3 to interact with TIF2. This same C-terminal region of PIAS1 has been shown to be involved in the interaction with STAT-1 and required for the inhibition of STAT-1-dependent gene activation [32]. The conserved region between amino acids 283 and 325 of PIAS3 contains a putative RING domain. In this context, it is of interest to note that recent reports have revealed that an intact RING domain in PIAS proteins is required for the ability of these proteins to function as E3-type SUMO-1 ligases [33]. Unlike ubiquitylation, sumoylation does not appear to promote protein degradation but rather was shown to be involved in establishing protein–protein interactions, subcellular compartmentalization and protein stability.

Biochemical studies indicate that PIAS proteins interact directly with different transcription factors, including STATs, p53 and steroid receptors, and can regulate their transcriptional activities both positively or negatively [13–15,34]. In particular, PIAS3 antagonizes the activity of STAT-3, inhibiting its DNA binding ability [18], and it can enhance or inhibit the transcriptional activity of androgen, glucocorti-

coid, progesterone and estrogen receptors differently depending on promoter, steroid receptor and cell type (this study and [14,15,17]). The mechanism underlying the ability of PIAS proteins to modulate transcription and, in particular, steroid receptor-dependent transcription is still unknown. The fact that PIAS3 was found to interact with TIF2 could suggest a potential mechanism of PIAS protein actions on nuclear receptor transcriptional activity through the interaction with nuclear receptor coactivators. In this regard, it will be interesting to investigate whether sumoylation plays a role in TIF2/PIAS3/nuclear receptor interaction and/or the function of its AF(s). Recently, the involvement of ARIP3/PIAS α in the sumoylation of the mouse homolog of TIF2, GRIP1, was reported [35–37]. Interestingly, our results demonstrate that the interaction between TIF2 and PIAS3 involved two surfaces of the TIF2 protein. One of these, encompassing the bHLH/PAS domain, has been reported to be also involved in the recruitment of p160 coactivators by TEF-4 transcription factors [38]. In contrast to TEF-4, PIAS3 binding can occur autonomously also through a second interface established with the central part of TIF2. This TIF2 region contains the nuclear receptor interacting domain that harbors three LXXLL motifs and the activation domain 1 or CBP-interacting domain. No relevant interaction was observed with the C-terminal part of TIF2 that contains the activation domain 2. Further studies will be necessary to determine whether both PIAS3-interacting domains are required for a functional interaction between TIF2 and PIAS3, or alternatively, both domains can interact independently, albeit with different efficiencies, with more than one molecule of PIAS3.

As has been described for other co-regulators of nuclear receptor coactivators, for example CARM-1 or Zac-1 [10,11], PIAS3 can act together with TIF2 to stimulate androgen-dependent transcription in an additive manner. It has been reported that PIAS3 can interact physically with AR [17]. Probably, the triple interaction between PIAS3, AR and TIF2 could help to stabilize and/or support the activity of the protein complex. However, it is also possible that the interactions of PIAS3 with AR and TIF2 in intact cells involve other protein(s) that may be part of a common complex. On the other hand, PIAS3 inhibits the activity of TIF2 for glucocorticoid- and progesterone-dependent transcription. As there is no evidence of direct interaction between PIAS3 and GR or PR, it is possible that in this case PIAS3 competes with GR or PR for TIF2 interaction. Differences in the affinities of NRs and PIAS3 for TIF2 may account for these different effects of PIAS3 on TIF2-mediated signalling.

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